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ORIGINAL ARTICLE

$CD8^+$ tumor-infiltrating lymphocytes predict favorable prognosis in malignant pleural mesothelioma after resection

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Abstract Defects in human leukocyte antigen (HLA) class I expression may allow tumor cells to escape immune recognition. T cell infiltration is associated with a good prognosis in many cancers. However, the role of HLA class I expression and tumor-infiltrating lymphocytes (TILs) in malignant pleural mesothelioma (MPM) has not been fully analyzed. In the present study, we investigated the immune profiles and conducted outcome analyses of MPM patients. HLA class I expression and TILs ($CD4^+$, $CD8^+$, and NK cells) were detected by immunohistochemistry in a series of 44 MPM cases. To detect HLA class I expression, specimens were stained with the anti-pan HLA class I monoclonal antibody EMR8-5. The expression of HLA class I was positive in all patients. There was no case that showed negative HLA class I expression. The density of $CD4^+$ and $CD8^+$ TILs were strongly correlated ($R = 0.76$, $p < 0.001$). A high density of $CD8^+$ TILs was a significantly better prognostic factor for the survival of patients with extrapleural pneumonectomy ($p < 0.05$). Multivariate analysis revealed that a high density of $CD8^+$ TILs is an independent prognostic factor for patients who underwent extrapleural pneumonectomy. The presence of intratumoral $CD8^+$ T cells was correlated with an improved clinical outcome, raising the possibility that $CD8^+$ T cells might play a pivotal role in the antitumor immune response against MPMs. Thus, the stimulation of $CD8^+$ lymphocytes might be an efficacious immunotherapy for MPM patients.

Keywords Malignant pleural mesothelioma · HLA class I · Tumor-infiltrating lymphocytes (TIL) · Immunohistochemistry · Immunotherapy

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Abbreviations

HLA	Human leukocyte antigen
TILs	Tumor-infiltrating lymphocytes
MPM	Malignant pleural mesothelioma

Introduction

The incidence of malignant pleural mesothelioma (MPM) is increasing worldwide as a result of widespread workplace exposure to asbestos that occurred in the past [1–3]. It has been predicted that 250,000 deaths in Europe and 103,000 deaths in Japan will be caused by MPM over the next 40 years [4]. MPM is a highly aggressive tumor. It has a poor prognosis, despite treatment efforts such as

extrapleural pneumonectomy [5], the new chemotherapeutic agent pemetrexed [6], and postoperative intensity-modulated radiotherapy [7]. To improve the prognosis of MPM, more effective therapeutic strategies, including the development of immunotherapy, are required. However, there is still uncertainty about the immune profiles in MPM, which is not an epithelial tumor.

Human leukocyte antigen (HLA) class I antigens are expressed on the surface of most human cells and present specific antigens. $CD8^+$ cytotoxic T lymphocytes can induce tumor killing upon direct recognition of tumor-specific antigens presented on HLA class I antigens on the surface of tumor cells.

Tumor-infiltrating lymphocytes (TILs) recognize tumor-specific antigens, thereby playing an important role in immune defense. In several cancers, the presence of TILs and the expression of HLA class I antigen are associated with prognosis [8–13]. In MPM, however, the role of TILs and HLA class I antigen is not well understood.

Here, we performed immunohistochemical assessment of HLA class I expression and TILs in patients with MPM. We also evaluated whether there is an association between immune profiles and clinical outcomes.

Materials and methods

Patients and specimens

Paraffin-embedded tumor specimens were obtained from 44 patients diagnosed with MPM between May 1997 and January 2008 in four institutes. This study was approved by the institutional review boards of each institute, and all patients provided written informed consent. Histopathological diagnoses were established by pathologists from each institute, and clinicopathological information was collected from patient charts. The TNM stage was based on the International Mesothelioma Interest Group classification [14]. A survival analysis of patients who underwent either curative extrapleural pneumonectomy or chemotherapy was conducted. Overall survival was defined as the time from the date of surgery or initiation of chemotherapy to death from any cause.

Immunohistochemistry

The following primary antibodies were used: anti-human HLA class I A, B, C (clone EMR8-5, Hokudo, Sapporo, Japan; 1:100 dilution), anti-human CD4 (clone 1F6, Novocastra, Newcastle, UK; 1:20 dilution), anti-human CD8 (clone C8/144b, DAKO, Glostrup, Denmark; 1:50 dilution) and anti-human CD56 (clone 1B6, Novocastra; 1:50 dilution). Tumor specimens were cut into sequential 5-μm-thick sections and deparaffinized by xylene and rehydrated by ethanol. Antigen retrieval was performed by autoclave heating at 121°C for 20 min in 10 nM citrate buffer (pH 6.0) for anti-human HLA class I and Tris–EDTA buffer (pH 9.0) for anti-human CD4, anti-human CD8, and anti-human CD56. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for anti-human CD4 and 3% hydrogen peroxide for anti-human HLA class I, anti-human CD8 and anti-human CD56. Then, tissue slides were incubated at room temperature for 1 h with anti-human HLA class I, anti-human CD4, or anti-human CD56; or, alternatively, the slides were kept at 4°C overnight with anti-human CD8. After incubation with the primary antibody, the streptavidin–biotin complex (SimpleStain MAX-PO kit, Nichirei, Tokyo, Japan) was used, followed by reaction with 3,3′-diaminobenzidine tetrahydrochloride–hydrogen peroxide as a chromogen and counterstaining with hematoxylin solution.

Evaluation of HLA class I and TILs

Expression of HLA class I was assessed by two investigators (N.Y. and E.K.) who were not informed of the patients' clinicopathological data. To evaluate HLA class I, the percentage of tumor cells with immunoreactivity on their membranes was calculated for at least five high-power (400×) fields. Average in the percentage of HLA class I positive cells were defined as frequency of HLA class I for each case.

To examine TILs, the number of cells per microscopic field (400×) with immunoreactivity to CD4, CD8 and CD56 were counted in five independent areas with the most abundant immunoreactive cells. We defined the average value of the five highest numbers in the slide as the number of TILs for each case. For analysis of association with prognosis, the patients were divided into two groups by the median number as the cutoff.

Statistical analysis

Data were analyzed with Pearson's chi-squared test. Densities of TILs were compared with the Student's t test. Survival probabilities were studied by the Kaplan–Meier method, and differences in survival time between patient subgroups were analyzed with a log-rank test. Uni- and multi-variate analyses were performed with the Cox proportional hazards model to estimate correlations between the immunohistological factors and overall survival. Statistical software (SPSS version 11.0.1; Chicago, IL) was utilized for all analyses. Statistical significance was established at the $p < 0.05$ level, and all analyses were two-sided.

Results

Patient characteristics

The median patient age was 59 years (range 35–85 years). There were 40 men and 4 women. More than 50% of patients had advanced-stage disease (stage III or IV). The disease stage (I–IV), histological diagnosis (epithelioid, biphasic, or sarcomatoid), and treatment (surgery, chemotherapy, or supportive care) are listed in Table 1.

HLA class I expression in MPMs

Representative images of immunohistochemical staining of HLA class I are shown in Fig. 1a, b. In addition, histogram of percentage of HLA class I expression is illustrated in Fig. 2. All patients had high expression of HLA class I in tumor cells; HLA class I expression was 100% in 33 patients (75%), and the lowest percentage of HLA class I expression was 70%.

Intratumoral lymphocytes in MPMs

Representative images of immunohistochemical staining of TILs are shown in Fig. 1c–e. The TIL counts are shown in Table 2. The densities of $CD4^+$ and $CD8^+$ TILs were strongly correlated ($R = 0.74$, and $p < 0.001$; data not shown). There was no correlation between the presence of intratumoral lymphocytes and major clinical features (data not shown).

Table 1 Patient characteristics ($n = 44$)

Characteristics	
Median age (range)	59 (35–85)
Men	40 (90.9%)
Women	4 (9.1%)
IMIG stage	
I	3 (6.8%)
II	17 (38.6%)
III	21 (47.7%)
IV	3 (6.8%)
Histology	
Epithelioid	26 (59.1%)
Biphasic	14 (31.8%)
Sarcomatoid	4 (9.1%)
Treatment	
Surgery	28 (63.6%)
Chemotherapy	9 (20.5%)
Best supportive care	7 (15.9%)

Association of lymphocyte infiltrates with clinical outcome

We analyzed the survival of 35 patients who underwent either surgical resection or chemotherapy. One patient who underwent surgical resection was excluded from the analysis because immunohistochemical staining of lymphocytes was not evaluable. Among the patients who received surgical resection or chemotherapy, 21 patients had epithelioid type, 10 patients had biphasic type, and 4 patients had sarcomatoid type, while disease stage was stage I in 1 patient, stage II in 12 patients, stage III in 20 patients, and stage IV in 2 patients. We found no significant differences in survival according to the density of $CD8^+$ cells (Fig. 3a). Next, we analyzed the survival of only the patients who underwent surgical resection. Among 27 patients who received surgical resection, 16 patients had epithelioid type, 7 patients had biphasic type, and 4 patients had sarcomatoid type; disease stage was stage II in 10 patients and stage III in 17 patients. Patients with a high density of $CD8^+$ cells (13 patients) had a significantly longer overall survival than those with a low density of $CD8^+$ cells (14 patients) ($p < 0.05$) (Fig. 3b). The prognosis of surgically treated patients with a high density of $CD4^+$ cells tended to be better than that of patients with a low density of $CD4^+$ cells ($p = 0.104$), although this tendency was not statistically significant (data not shown). There were no significant differences in survival according to the density of $CD56^+$ NK cells (data not shown).

In the univariate analysis for the patients who underwent extrapleural pneumonectomy, histology (i.e., epithelioid) and $CD8^+$ TILs (i.e., high density) were identified as significant prognostic factors. Furthermore, multivariate analysis revealed that a high density of $CD8^+$ TILs was an independent prognostic factor in the population (hazard ratio 0.27, 95% CI 0.09–0.83; Table 3).

Discussion

This study assessed both the HLA class I expression and intratumoral T lymphocytes in MPM. We found that the expression of HLA class I antigen was maintained in all of the examined MPMs. Notably, the presence of $CD8^+$ tumor-infiltrating lymphocytes was correlated with the survival of patients who underwent extrapleural pneumonectomy. Taken together, these results demonstrate that $CD8^+$ T cells may adequately recognize the tumor-specific antigens presented by HLA class I antigens in MPM and effectively kill early-stage mesothelioma cells.

Tumor-infiltrating lymphocytes (TILs) are found in cancer tissues. The abundance of tumor-infiltrating $CD8^+$ cells correlates with a good prognosis in colon cancer [8],

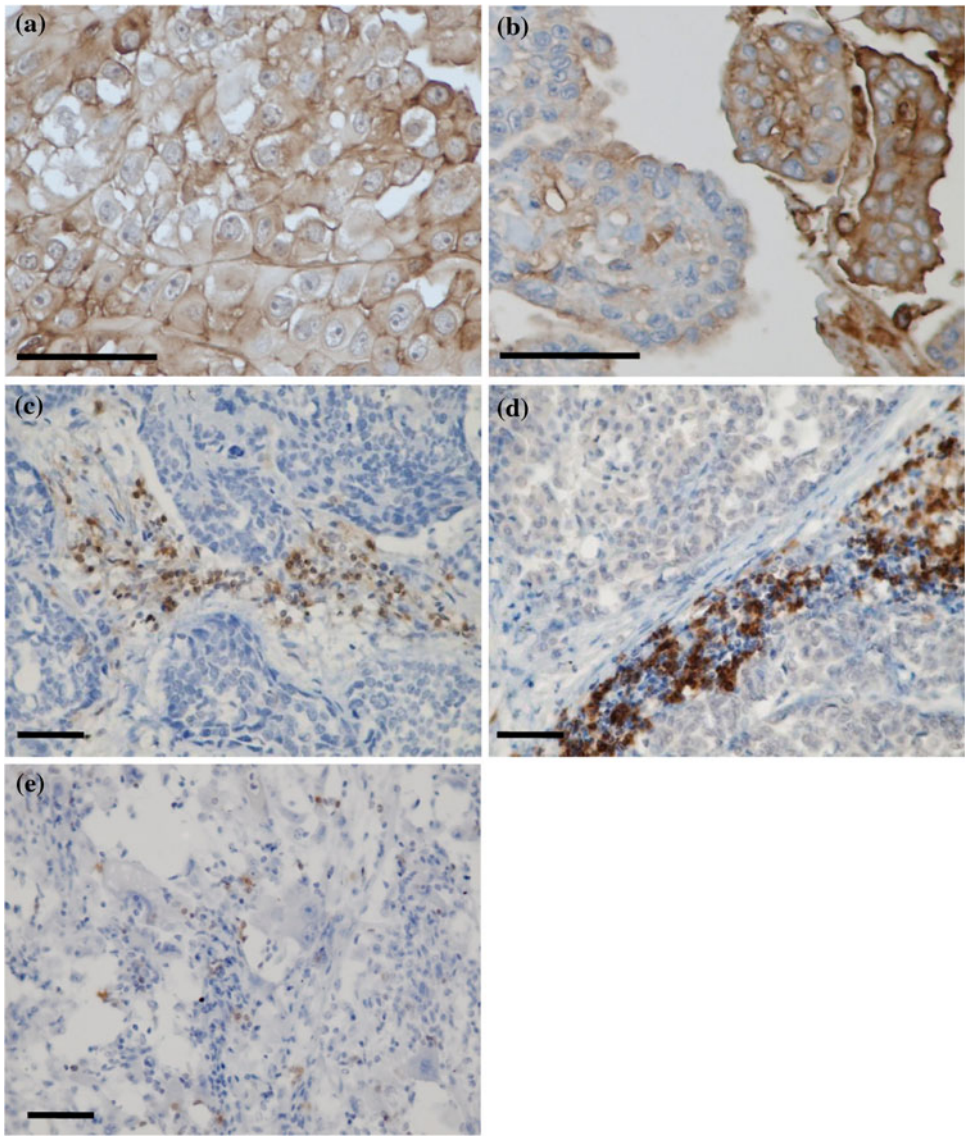

Fig. 1 Representative images of immunohistochemical staining of **a** 100% positive expression of HLA class I, cell membranes of tumor cells are completely stained; **b** 70% positive expression of HLA class I; **c** anti-CD4 antibody; **d** anti-CD8 antibody; and **e** anti-CD56 antibody (*scale bar* 50 μm)

esophageal cancer [15, 16], ovarian cancer [17], and hepatocellular carcinoma [18]. In renal cell carcinoma [19] and lung cancer [13, 20], however, tumor-infiltrating $CD8^+$ cells were not associated with prognosis. Harlin et al. [21] have recently reported that high expression of CXCR3 and CCR5 ligand chemokines generated from tumor are important to promote migration of $CD8^+$ effector cells. Differential expression of these chemokines could lead to differential frequency of lymphocytes infiltrate, which might affect prognosis of the patients. In addition, the origin and stage (early or advanced) of the tumor or subsequent therapy against the disease might account for the discrepancy of prognosis.

Therefore, we analyzed survival in a homogenous population of patients who had resectable MPMs and underwent extrapleural pneumonectomy. Gao et al. [18] reported that the intratumoral balance of regulatory and cytotoxic T cells is an independent predictor of recurrence and survival after resection of hepatocellular carcinoma. The immune defense elucidated by cytotoxic T cells might be more effective in the operable tumors which correspond to the earlier stages of the disease. Cytotoxic T cells (or reactivated T cells from memory phase) are expected to suppress the initial recurrence or metastasis after surgical treatment.

Some studies have assessed TILs in MPM [22–24]. Leigh et al. [22] reported that the presence of significant lymphoid infiltration indicates a better prognosis for longer survival. However, the TILs and their subsets were analyzed by using hematoxylin–eosin staining, but not by immunohistochemical staining. Mudhar et al. [23] analyzed the TILs in MPM by immunohistochemical staining and reported no association between survival and the prominence of the infiltrate of pan leukocytes, T cells, or NK cells in 15 MPM cases. Recently, Anraku et al. [24]

reported that the presence of high levels of $CD8^+$ tumor-infiltrating lymphocytes was associated with a better prognosis in patients undergoing extrapleural pneumonectomy. Although the results from these previous reports vary, they have shed light on the importance of T lymphocytes for antitumor immunity in MPM, which is consistent with our results.

The expression of HLA class I antigen is totally lost or downregulated in many types of tumors. Marincola et al. [25] reported that the frequency of the total loss or downregulation of HLA class I ranged from 31 to 70% among various types of tumors. In lung cancer, the frequency of total loss ranged from 27 to 80.5%, and the frequency of the downregulation of HLA class I ranged from 13.2 to 35.4% [13, 26–28]. In the present study, the expression of HLA class I was positive in all MPM patients, whereas there was no case that completely lacked HLA class I expression. This finding indicates that MPM might be a type of tumor in which HLA class I expression is relatively maintained. The high HLA class I expression in the present study may be attributed to the novel antibody EMR8-5, which can react with the heavy chains of all alleles of HLA-A, B and C in formalin-fixed, paraffin-embedded tissue sections [29]. Another antibody, HC10, has been used in many previous studies; this antibody preferentially reacts with HLA-B and C but weakly reacts with HLA-A.

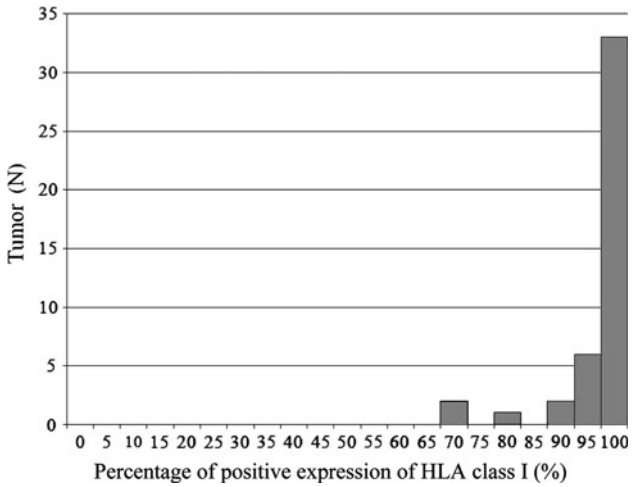

Fig. 2 Histogram of percentage of HLA class I expression; 100% of HLA class I expression in 33 patients (75%), 95% in 6 patients (14%), 90% in 2 patients (4%), 80% in 1 patient (2%), and 70% in 2 patients (4%)

Table 2 The numbers of TILs per slide

	Mean ± SD	Range	Median
$CD4^+$ TILs	51.1 ± 41.8	0.2–159.7	37.3
$CD8^+$ TILs	103.3 ± 106.9	8.8–547.5	64.5
$CD56^+$ TILs	5.4 ± 8.3	0.0–41.8	1.8

Past studies assessed Ki-67 [20] and granzyme B [30]; activation markers of $CD8^+$ TILs and reported the association between these activation markers and prognosis. Helper $CD4^+$ cells play a pivotal role in the activation and expansion of $CD8^+$ T cells [31]. In contrast, regulatory T cells which express the $CD4^+$, $CD25^+$ and Foxp3 phenotype are found in the tumor microenvironment and are thought to dampen T cell immunity [32]. The activation markers of $CD8^+$ T cells and phenotype of $CD4^+$ T cells were not evaluated in the present study. However, infiltration of $CD8^+$ T cells predicted favorable prognosis and there was the strong correlation between frequency of $CD8^+$ and $CD4^+$ T cells, suggesting that both type of cells would interact each other and subsequently orchestrate antitumor immune response.

In conclusion, we found that all of the MPM patients in our study were positive for the expression of HLA class I antigen and that a high density of $CD8^+$ TILs was a significant prognostic factor for longer survival in surgically resected MPMs. These results suggest that $CD8^+$ TILs have an important role in antitumor immunity of patients with MPM and that the stimulation of $CD8^+$ lymphocytes can be a potential therapeutic strategy for the disease. Although several immunotherapies for MPM, intralesional granulocyte–macrophage colony-stimulating factor infusion [33], intrapleural interleukin-2 [34], interferon alpha

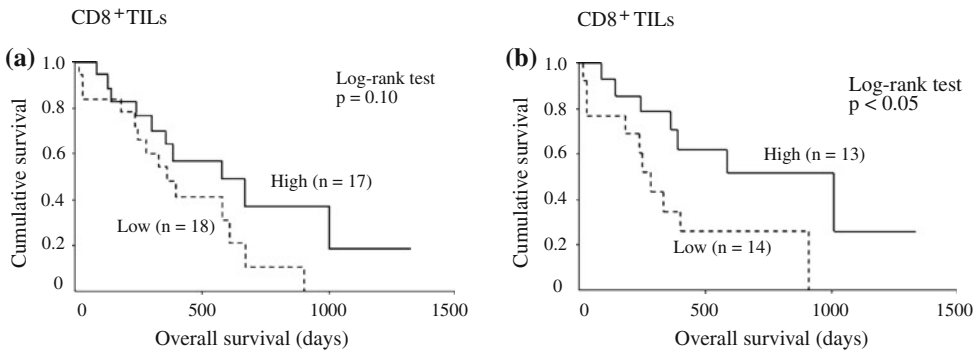

Fig. 3 Kaplan–Meier curves show overall survival after treatment (extrapleural pneumonectomy or chemotherapy) according to the density of $CD8^+$ TILs (**a**), and overall survival after extrapleural pneumonectomy according to the density of $CD8^+$ TILs (**b**)

Table 3 Univariate and multivariate analysis with the Cox proportional hazards model for patients who underwent extrapleural pneumonectomy

Variable	Univariate		Multivariate	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Histology				
Epithelioid versus non-epithelioid	0.24 (0.08–0.7)	<0.01	0.19 (0.06–0.6)	<0.01
Age (years)				
≥65 versus <65	1.18 (0.4–3.49)	0.76		
Sex				
Women versus men	1.23 (0.27–5.58)	0.79		
Stage				
III + VI versus I + II	1.48 (0.52–4.2)	0.47		
$CD4^+$ TILs				
High versus low	0.42 (0.14–1.24)	0.12		
$CD8^+$ TILs				
High versus low	0.34 (0.12–0.99)	<0.05	0.27 (0.09–0.83)	<0.05
$CD56^+$ TILs				
High versus low	0.66 (0.25–1.78)	0.41		

HR hazards ratio, *CI* confidence interval

[35], and recombinant anti-mesothelin immunotoxin SS1P [36, 37] have been tested, none of them are available for clinical practice. Further investigations on immunological function or specific antigens are warranted to develop robust immunotherapies against MPM.

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